1   **Comparison of phenotypic and genotypic methods for the detection of ESBLs and AmpC**

2   **producing *Pseudomonas aeruginosa* isolates from intensive care units**

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6   **Abstract**

7   Detection of AmpC and ESBL producing *P. aeruginosa* by phenotypic methods is challenging,

8   especially in low-income countries such as Pakistan. Therefore, a molecular method was

9   developed for rapid detection of these resistance markers. A total of 303 clinical samples were

10   collected from intensive care units (ICUs) of the Jinnah postgraduate medical centre (JPMC)

11   Karachi, Pakistan. The isolates were identified by traditional and matrix-assisted laser desorption

12   ionization–time-of-flight mass spectrometry (MALDI-TOF-MS). Isolates were phenotypically

13   analyzed for AmpCs and ESBL by D-test and by double disc synergy, respectively. The Check

14   MDR CT103 XL and PCR techniques were used for the detection AmpCs and ESBLs. Out of 303

15   isolates, 148 (48.8%) were *P. aeruginosa*. The resistance pattern of *P. aeruginosa* against

16   piperacillin, cefatizidime and cefepime was 59.4%, 64.8% and 59.4% respectively. More than

17   60% isolates were resistant to aminoglycosides and ciprofloxacin. All (148) strains were found

18   sensitive to colistin. Phenotypic ESBL prevalence was 8.8% whereas genotypic resistance was

19   29.1%. bla_{VEB} was the most prevalent ESBL. Although 25.67% of *P. aeruginosa* isolates were

20   positive phenotypically for AmpC, microarray (Check-MDR) analysis did not detect

21   chromosomally located AmpC in any of the isolates.

22   **Abbreviations**

Bla, β-lactamase; CPs, Carbapenemases; DDST, Double disc synergy test; ESβLs, Extended spectrum β-lactamases; HAP, Hospital-acquired pneumonia; ICUs, Intensive care Units; MALDI-TOF-MS, Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry; MDR, multi-drug resistant; pAmpCs, Plasmid mediated AmpCs; PDR, Pan-drug resistant; XDR, Extensively drug-resistant

Key words

Antibiotic resistance, D-test, Intensive Care Units, Microarray, *Pseudomonas aeruginosa*

1. Introduction

Pseudomonas aeruginosa commonly causes infections among the immunocompromised patients admitted in intensive care units (ICUs) [1]. The emergence of multi-drug resistant (MDR), extensively drug-resistant (XDR) and pan-drug resistant (PDR) strains of *P. aeruginosa* has serious consequences including therapeutic failure, increase in healthcare cost by 70%, increase in the rates of morbidity and mortality and prolonged hospital stay [2]. The MDR, XDR and PDR strains have evolved through genomic plasticity and accumulation of selected mutations in chromosomal genes and transmission of exchangeable resistance elements [1]. Indeed, the overexpression of the chromosomal AmpC and ESBL production are the two primary mechanisms of resistance against antipseudomonal cephalosporins, penicillins and other β-lactam antibiotics [3, 4]. The bla_{AmpC} genes, usually located on chromosome but plasmid mediated genes are clinically important [5]. The most common types of plasmid mediated AmpC (pAmpCs) are CMY, ACT, DHA, FOX and MIR in *P. aeruginosa* [5, 6, 7].

44 Commonly encountered ESBLs are PER, GES, VEB, BEL and PME of class A, while TEM,

45 SHV and CTX-M-type appear less frequently in *P. aeruginosa* [8, 9, 10,11].

46 Clinical and Laboratory Standards Institute (CLSI) does not recommend the commonly

47 employed phenotypic methods (double disc diffusion test with clavulanic acid and D-Tests)

48 for the detection of ESBLs and AmpCs in *P. aeruginosa* [12]. Yet these phenotypic methods

49 continue to be used in resource limited settings. A molecular method, Check-MDR CT103 XL

50 (Check-points, Wageningen, Netherlands), has been developed as a rapid and accurate assay

51 for the detection of clinically relevant ESBLs, pAMPCs and carbapenemase variants in a single

52 tube reaction [13]. The prevalence of ESBL-producing strains varies geographically [2] and

53 the prevalence of ESBL and AmpC producing *P. aeruginosa* is partially described in Pakistan.

54 In the present study we highlight the compromised sensitivity of phenotypic assays used for

55 the determination of ESBL and AmpC types in *P. aeruginosa* as compared to DNA microarray

56 (Check-MDR) and PCR.

57 **2. Materials and Methods**

58 *2.1* **Setting**

59 The present work was performed in the Department of Microbiology, Basic Medical

60 Sciences Institute (BMSI), Jinnah Postgraduate Medical Centre (JPMC) Karachi, Pakistan (a

61 teaching hospital with >1500 beds) in collaboration with the Department of Microbiology

62 University of Karachi, Dow University of Health Sciences, Karachi, and the UCL Centre for

63 Clinical Microbiology, Royal Free Campus, London.

64 *2.2* Sample size

65   A total of 303 clinical specimens, that were growth positive, were processed from the

66   patients admitted to ICUs at JPMC, Karachi, Pakistan during November 2015 to May 2016. The

67   duplicated/repeated, Glucose fermenting Gram-negative bacilli and Gram-positive cultures were

68   excluded. The specimens were collected in accordance with the standard operating procedures

69   [14]. The informed consent was obtained from the patient or attendant and ethical approval was

70   taken from the institutional review board (IRB), JPMC Karachi (No. F.2-18/2014-

71   GENL/31649577/JPMC).

72   *2.3* Microbiological assays

73   Clinical specimens (tracheal aspirates, urine, blood, sputum, pus) were processed using

74   standard techniques and blood specimens were directly inoculated in blood culture bottles

75   according to the standard protocols [14]. *P. aeruginosa* isolates (148) were selected for further

76   analysis.

77   *2.4* Identification of *P. aeruginosa*

78   Isolates were initially identified by routine cultural characteristics and battery of

79   biochemical tests. The oxidase test was performed by Microbact™ oxidase strips (Oxoid, UK)

80   [15]. These provisionally identified isolates were confirmed by the API 20 NE (Biomerieux, Marcy

81   I'Etoile France) and re-confirmed by the matrix-assisted laser desorption ionization–time-of-flight

82   mass spectrometry (MALDI-TOF-MS, Microflex, Bruker Daltonics) at the Centre for Clinical

83   Microbiology, UCL, London, UK.

84   *2.5* Antibiotic susceptibility testing (AST)

85   AST was determined by the disk diffusion technique using Mueller-Hinton agar (MHA), (Oxoid,

86   UK) according to the recommendations of the CLSI. AST results were checked and compared with

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87 the results of micro-broth dilution method and Etest as recommended by CLSI. *P. aeruginosa*

88 ATCC 27853 was used as a quality control strain [12].

89 *2.6* Phenotypic detection of AmpCs and ESBLs in *P. aeruginosa*

90 AmpCs in *P. aeruginosa* was determined by a diffusion test, D-test [16]. Cabapenem

91 [imipenem (IPM), meropenem (MEM) and amoxicillin-clavulanate (AMC)] discs were used as

92 inducers, whereas, antipseudomonal cephalosporins and penicillin [ceftazidime (CAZ),

93 piperacillin (PIP), and aztreonam (ATM)] were applied as substrate antibiotics (Oxoid, UK) as

94 recommended by the CLSI for AST of *P. aeruginosa* [12]. After incubation at $35^{\circ}C$ for 16-18

95 hours, the growth inhibition zones were determined. A difference of ≥ 2 mm at inducer side and

96 non-inducer side was interpreted as AmpCs positive strain.

97 The ESBLs were phenotypically determined by the double disc synergy test (DDST) with

98 clavulanic acid (CA) in accordance with Laudy et al. [11] and CLSI recommendations [12]. The

99 shoulder formation towards the AMC and any of CAZ, CRO, FEP, or ATM was interpreted as

100 ESBLs producer.

101 *2.7* Detection of β-lactamases by DNA microarray

102 The ESBLs and AmpCs were determined by the Check-MDR CT103XL microarray kit (Check-

103 Points Health BV, Wageningen, Netherlands). This assay was performed at the Centre for Clinical

104 Microbiology, Royal Free Campus, London, UK by following the manufacturer's instructions. The

105 principle of this technique is based on the multiplex ligation detection reaction (LDR). It included

106 two probes for the targeted resistance genes which make it superior to multiplex PCR. This

107 microarray targeted various ESBL and AmpC genes including BEL

108 CTX-M-groups, GES-ESβL, PER, SHVs, TEMs, VEB variants and ACC, ACT, CMY, DHA,

109 FOX, MIR, MOX respectively [13].

110   *2.8* Detection of *bla*PER and *bla*VEB genes by PCR

111   The primers for the PCR were designed according to the microarray results. PCR was

112   performed using G-Strom-482, (Gene Technologies Ltd, UK) thermocycler and the amplification

113   kit (Qiagen, Germany). PCR reaction cycles and the annealing temperature for VEB gene have

114   been described previously [17]. The optimized PCR cycle time used in this work was 15, 0.5, 0.5,

115   1 and 10 min for initial, denaturation, annealing, extension and final extension respectively at the

116   different temperature (Table.1).

117   Table 1 The primers and their cycles temperature in this work

Gene	Primer Sequence	*In. (°C)	*Det. (°C)	*An. (°C)	*Ext. (°C)	*FE. (°C)	Size (bp)
*bla*VEB	F- GTTAGCGGTAATTTAACCAGATAG	95	94	55	72	72	1070
	R- CGGTTTGGGCTATGGGCAG						
*bla*PER	F- GCTCCGATAATGAAAGCGT	95	94	53	72	72	520
	R- TTCGGCTTGACTCGGCTGA						

118   *In: Initial Temperature, Det: Denaturation, An: Annealing temperature, Ext: Extension, FE: Final
119   Extension

120   *2.9* Data analysis

121   The initial data was recorded in Microsoft Excel version 2010 and statistical analysis was
122   conducted using SPSS (Statistical Package for Social Sciences, Microsoft Inc., USA) software,
123   version 16.0 for Windows. The sensitivity and specificity of phenotypic assays were calculated by
124   using the web calculator (https://www.medcalc.org/calc/diagnostic_test.php).

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126   **3. Results**

127   *3.1* Frequency of Gram-negative Bacilli

128   Out of 303 samples 154 (50.8%) were positive for *Pseudomonas* spp., 108 (35.6%) for

129   *Acinetobcter baumannii*, and 41 (13.5%) for other Gram-negative bacteria. *P. aeruginosa* was the

130   most prevalent species amongst Pseudomonads (148/154, 96.1%); most of the isolates were

131   obtained from respiratory (61/148, 41.2%) and blood specimens (53/148, 35.8%), followed by pus

132   samples (Table.2).

133   *3.2* Antibiogram of *P. aeruginosa*

134   *P. aeruginosa* isolates were phenotypically resistant to most of the antipseudomonal penicillin and

135   cephalosporins but a significant proportion was sensitive to piperacillin/tazobactam (PZT)

136   (58/148, 39.2%). Resistance against the carbapenems (imipenem 74.3%, meropenem, 68.2%),

137   ciprofloxacin (65.5%) and aminoglycosides (64.2%) was also higher (Table.3). All the *P.*

138   *aeruginosa* isolates were resistant to ampicillin, ampicillin sulbactam and ceftriaxone.

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140 Table 2 Frequency of *P. aeruginosa* in relation to the specimens (n=148)

Specimen	Disease	*P. aeruginosa*	Percentage %
Tracheal aspirates	VAP/HAP[a]	61	41.2
Blood	Septicemia	53	35.8
Pus	Diabetic	18	12.2
	Foot/postsurgical wound		
Urine	UTIs	11	7.4
Sputum	Pneumonia	5	3.4
Total		148	100

141 [a]Ventilator-associated pneumonia, Hospital-acquired pneumonia, [b]Urinary tract infections

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151   Table 3 Susceptibility patterns of *Pseudomonas aeruginosa* by combined disc diffusion method

152   and Etest, according to the recommendation of CLSI (n=148)

Antibiotics class	Number of isolates (%)			Total (%)
	Resistant	Intermediate	Sensitive	
Penicillins				
Piperacillin (PIP)	88 (59.45)	8(5.40)	52(35.13)	148(100)
Piperacillin/tazobactam (PZT)	58(39.18)	11(7.43)	79 (53.37)	148(100)
Cephalosporins 3rd Generation				
Ceftazidime (CAZ)	96 (64.86)	2(1.35)	50(33.78)	148(100)
Cephalosporins 4thGeneration				
Cefepime (FEP)	88 (59.45)	-	(40.54)	148(100)
Carbapenems				
Imipenems (IPM)	110(74.32)	4(2.70)	34(22.97)	148(100)
Meropenems (MEM)	101(68.24)	2(1.35)	45(30.40)	148(100)
Aminoglycosides				
Amikacin (AK)	96 (64.86)	3(2.02)	49(33.10)	148(100)
Gentamicin (CN)	95(64.18)	5(3.37)	47(31.75)	148(100)
Tobramycin (TOB)	95(64.18)	5(3.37)	47(31.75	148(100)
Quinolones				
Ciprofloxacin (CIP)	97(65.54)	3(2.02)	48(32.43)	148(100)
Lipopetides (Polymyxins)				
Colistin (CT)*	-	-	148(100)	148(100)

153   *Broth microdilution, CLSI (2018) guidelines

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155   *3.3* Phenotypic and genotypic detection of ESBLs and AmpCs

156   Phenotypic assessment identified 8.8% (13/148) isolates of *P. aeruginosa* as ESBL

157   producers, whereas the genotypic prevalence of ESBLs was 43/148 (29.05%). *bla*$_{VEB}$ was the most

158   common marker (Fig.1). The microarray method (Check-MDR) did not detect AmpC producers;

159   however, 38 (25.67%) isolates were positive for AmpC by the D-test (Fig. 2).

160   *3.4* Sensitivity and specificity of phenotypic methods

161   The sensitivity and specificity of the double disc synergy method for ESBLs in *P.*

162   *aeruginosa,* was 30.2% and 100%, respectively, compared to the microarray (Check-MDR). The

163   data was also analyzed for the co-production of AmpC in genotypically VEB positive and 11.6%

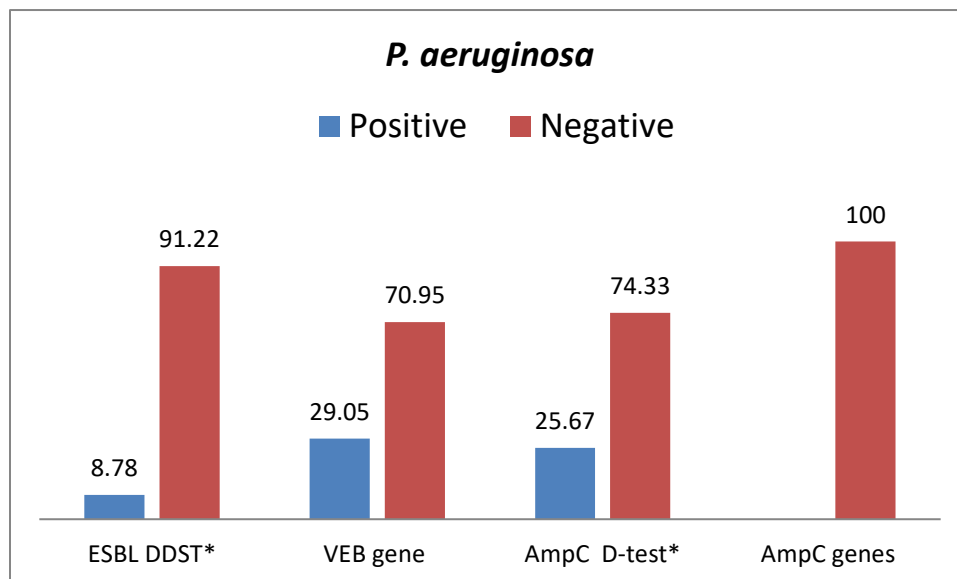164   (5/43) isolates were found D-test positive for AmpC.

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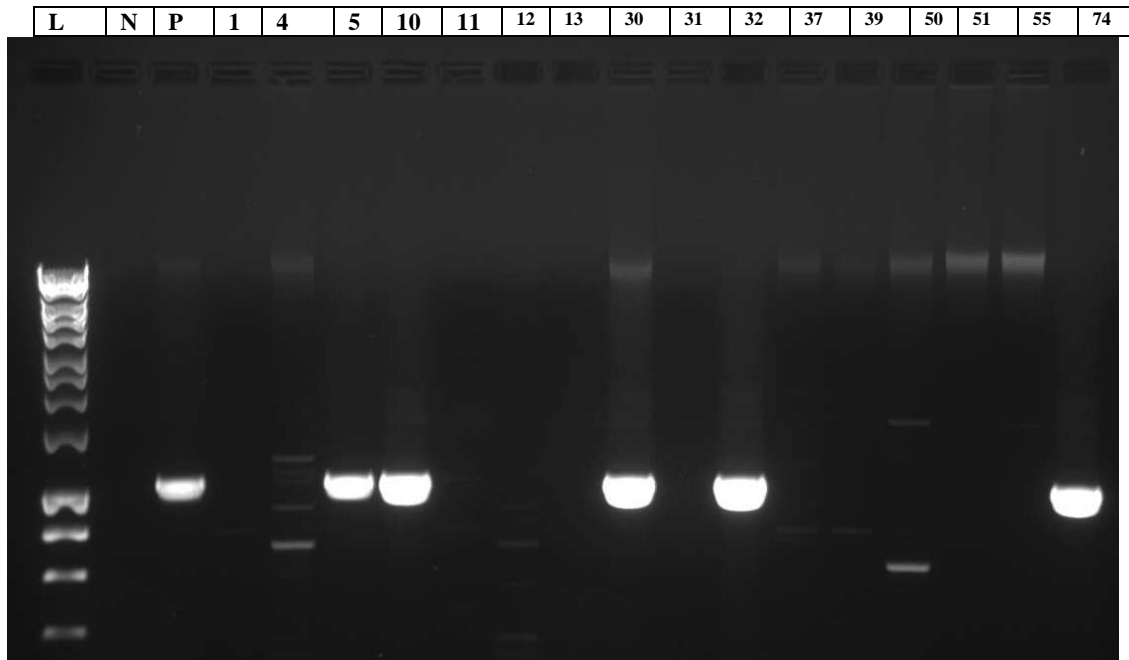
Figure 1 PCR results of *bla*$_{VEB}$ gene (1070 bp), L, DNA ladder (1kb), N negative control, P, positive
control, sample #. 5, 10, 30, 32,74 were positive and 1, 4, 11, 12, 13, 31, 37, 39, 50, 51, and
55 were negative

Figure 2 Phenotypic and genotypic compression of ESBLs and AmpC in *P. aeruginosa*

4. Discussion:

The environment in ICUs favors survival of *P. aeruginosa* due to its ability to grow in nutritionally poor environments, resistance to commonly used disinfectants and other environmental factors such as favorable temperature and humidity. This increases the risk of transmission of multidrug resistant and carbapenem resistant *P. aeruginosa* to immunocompromised and critically ill patients [18, 19, 20]. The present study corroborated previous findings that the resistance to imipenem and meropenem was more prevalent than the antipseudomonal penicillins (piperacillin and piperacillin/tazobactam) and cephalosporins including ceftazidime and cefepime in *P. aeruginosa* [21]. The resistance may be attributed to the alteration of porins or repression of OprD, presence of intrinsic AmpCs and over-expression of MexAB-OprM and modification in efflux pump activity [22, 23]. Among these phenotypically tested strains, AmpCs producing *P. aeruginosa* showed higher prevalence (26.0%) than ESBLs (8.8%) producers in present study. However the results of microarray (Check-MDR) were in contrast to the phenotypic results, showing the higher prevalence of ESBL producing *P. aeruginosa.* A similar pattern of ESBLs and AmpC has been reported from Pakistan but at lower frequencies [24]. Lower prevalence of ESBLs (7.4%) in *P. aeruginosa* has also been found in other countries, such as in Egypt [13]. The AmpCs were not found by the microarray (Check-MDR) in all the *P. aeruginosa* isolates as the microarray (Check-MDR) detects only plasmid mediated AmpC (pAmpCs). Previously microarray (Check-MDR) has been reported as 100% specific and sensitive for pAmpC targets [13].

VEB-1 was initially discovered in *E. coli* from Vietnam (1996), and then it was widely reported in *Pseudomonas* spp. [25]. In the present study, VEB appeared as the only ESBL in *P. aeruginosa* (28.4%) and it is may be endemic in this geographic location. Other variants of ESBLs

198 (CTX-M-15 and OXA-10) have also been reported from this region [26]. The ESBL in *P.*

199 *aeruginosa* is not restricted to some types but it varies from VEB to OXA-like, GES, PER and

200 CTX from various geographic regions [11, 15, 26, 27]. The higher frequency of *bla*$_{VEB}$ carrying

201 *P. aeruginosa* in this study may be due to clonal spread in the ICUs of this hospital or these strains

202 are present in environment. Woodford et al. also found higher prevalence of VEB harboring *P.*

203 *aeruginosa* from the UK [25].

204   In summary, the sensitivity of phenotypic methods is compromised due to the accumulation

205 of different resistance mechanisms against the extended cephalosporins, which are used as

206 substrate in the phenotypic test for ESBLs detection.

207 **5. Conclusion**

208   The phenotypic methods for the detection of ESBLs and AmpC have good specificity but

209 poor sensitivity than genotypic tests. The VEB-type producing *P. aeruginosa* strains are common

210 in this hospital. Other than ESBLs, resistance mechanisms are present in the *P. aeruginosa* which

211 may be investigated at local level to understand the molecular mechanisms of resistance.

212 **Declarations**

213 **Consent for publication**

214 Not applicable

215 **Availability of data and materials**

216 The datasets used and/or analysed during the current study are available from the corresponding

217 author on reasonable request.

218 **Competing interests**

225   **References**

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